IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of

Hacker et al. Group Art Unit: 1636

Application No. 10/525,558 Examiner: Michele Joike

Priority claimed from: PCT/EP2004/006886 (filed June 25, 2004) and German application 103 28 669.1 (filed June 26, 2003)

Filed: September 2, 2005

For: Plasmid-Free Clone of *E. Coli* Strain DSM6601

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March 16, 2011

BRIEF ON APPEAL

Hon. Commissioner of Patents
and Trademarks

Sir:

In connection with this patent application, Appellant submits this Brief on Appeal.

i. Real Party in Interest

The real party in interest is the assignee, Pharma-Zentrale GmbH.

ii. Related Appeals and Interferences

There are no related appeals or interferences.

iii. Status of the Claims

Claims 1-6 are pending in the application.

Claims 7 and 8 have been cancelled.

Claims 1-6 stand finally rejected, and are on appeal here.

iv. Status of the Amendments

No amendments were filed after the October 19, 2010 final rejection.

v. Summary of the Claimed Subject Matter

Independent claim 1 recites a plasmid-free clone of Escherichia coli strain DSM 6601, which strain is identified as having accession number DSM 16700. (In the document labeled "Substitute Specification, Clean Version", see page 2, lines 4-5; page 3, lines 24-27; Figure 1; and the Amendment to the Specification dated February 24, 2010, which provide deposit information for this strain).

Claim 2 recites the method of preparing the plasmid-free clone, comprising the following steps:

a) introducing a resistance gene into plasmids pMut1 and pMut2, (In the document labeled "Substitute Specification, Clean Version", see Figure 1; page 2, line 20 – page 3, line 4)

b) introducing the *sacB* gene into the plasmids obtained in step a) so as to produce a pMut1 plasmid carrying a resistance gene and a *sacB* gene, and a pMut2 plasmid carrying a resistance gene and a *sacB* gene, (In the document labeled "Substitute Specification, Clean Version", see Figure 1; page 3, lines 6-7)

c) introducing the altered pMut1 and pMut2 plasmids obtained in step b) into the Escherichia coli strain DSM 6601, and cultivating the strain obtained thereby under conditions in which the naturally occurring pMut1 and pMut2 plasmids are displaced by the altered pMut1 and pMut2 plasmids obtained in step b); (In the document labeled "Substitute Specification, Clean Version", see Figure 1; page 3, lines 8-17)
and

d) cultivating the clones obtained in step c) that substantially only permit the growth of bacteria that lack the *sacB* gene, so that the Escherichia coli containing the altered pMut1 and pMut2 plasmids do not grow, and thereby is produced a plasmid-free clone of Escherichia coli strain DSM 6601. , (In the document labeled "Substitute Specification, Clean Version", see Figure 1; page 3, lines 18-27)

vi. Grounds of Rejection to be Reviewed on Appeal

1. Whether claim 1 is patentable under 35 U.S.C. §103(a) over Uraji et al. in view of Blum-Oehler et al (WO 99/44134) in view of Trevors et al., and further in view of Gasson et al.

2. Claims 2-6 are patentable under 35 U.S.C. §103(a) over Uraji et al. in view of Blum-Oehler et al (WO 99/44134) in view of Trevors et al., and Gasson, and further in view of Alexeyev et al.

vii. Argument

1. Issue No. 1: Whether claim 1 is patentable under 35 U.S.C. §103(a) over Uraji et al. in view of Blum-Oehler et al (WO 99/44134) in view of Trevors et al., and further in view of Gasson et al. Appellant respectfully asserts that it is.

There are two remaining obviousness rejections of the claims, referenced above in section (vi), both citing overlapping art. The four overlapping references are (1) Uraji, (2) Blum-Oehler, (3) Trevors, and (4) Gasson. The Examiner cites Uraji for teaching a method to cure a single plasmid from a gram negative bacteria – not the DSM 6601 strain or any *E. coli* strain, or the pMut plasmids, or curing more than one plasmid. Blum-Oehler is cited for the disclosure of Nissle 1917 (DSM 6601), which is not plasmid-free but contains two plasmids (pMut1 and pMut2). The Examiner cites Trevors et al. for describing curing methods for removing bacterial plasmids. Gasson et al. is cited for teaching the curing of five plasmids from a *Streptococcus* strain.

The Examiner acknowledges that none of these references teaches a plasmid-free clone of DSM 6601 or even the desirability of acquiring such a clone.

"The Examiner agrees that none of the references individually teach the curing of the strain DSM 6601 or the desire to cure pMut1 and pMut2; that's why she used 4-5 references." (October 19, 2010 Office Action, page 4)

We respectfully submit that the Examiner has failed to present a prima facie case of obviousness because there is no motivation to modify the Blum-Oehler reference to acquire a clone that is free of both plasmids pMut1 and pMut2. No reference teaches the curing of DSM 6601 or the desire to cure it of both pMut1 and pMut2. In addition, for

the reasons enumerated below, even if a prima facie case is deemed presented, Appellants believe it is rebutted by the facts of the references.

The primary reference **Uraji** is itself quite irrelevant to claim 1 on many points, which is pointed up by the string of secondary references cited to make up for its deficiencies. Uraji et al. describes the removal of plasmids in a bacterium via introduction of the sacB gene and a kanamycin-resistant cassette. The bacterium already contains a second plasmid without sacB gene. The plasmids are removed by cultivation of the bacterium on a culture medium containing saccharose and then on one with kanamycin.

On page 5 of the Office Action dated April 30, 2008, the Examiner has noted that Uraji does not teach the sacB gene on the second plasmid (since it is already present in the cell) – however, the Examiner suggested that Uraji does

> "teach that sacB should be in a plasmid being introduced into the cell that is being cured. Therefore, if two plasmids are being introduced into the cell, it would follow that both plasmids would contain the sacB gene in order to cure the cell. In claim 6, the steps are the reverse of the method steps taught by Uraji et al. First, the transformed bacteria are cultivated on plates containing the antibiotic, and then subsequently on plates containing saccharose."

MPEP 2144.04(IV)(C) is cited.

However, a simple derivation of the method of Uraji (alone or combined with the other cited art) would not produce the claimed modified *E. coli* DSM 6601. In view of Uraji's teachings of the transformation of the plasmids, simply changing the order of the culture mediums with the order selected in the application would not be obvious or successful. The Uraji article deals with the bacterium *Agrobacterium* and the removal of pTi plasmids. These plasmids are tumor-inducing and can be simply transferred to plants by *agrobacterium* where in addition to the creation of tumors, these plasmids simultaneously produce nutrients for the bacteria. For these reasons the removal of plasmids from the bacterium is desirable.

Notably, Uraji describes curing techniques only for *Agrobacterium* and not for *E. coli*. Uraji teaches carefully that its curing techiques are designed for use in plasmids found in *Agrobacterium* bacterium groups. (See Abstract of Uraji et al.). From the

standpoint of someone having ordinary skill in this art, *Agrobacterium* is neither genetically, physiologically or microecologically comparable to the probiotic, non-pathogenic *E. coli* strain DSM 6601, which is used in humans to treat gastrointestinal dysfunctions and diseases. *Agrobacterium* can only infect and colonize plants; *E. coli* is a bacterium that populates the human and animal intestine. *Agrobacterium* with its pTi plasmid growths acts to the detriment of plants; *E. coli* has a positive impact on the host organism through different effects, such as the production of Vitamin K, the constant training of the mucosa immunological system and the production of secretory immunoglobulin A. *Agrobacterium* and *E. coli* are two completely different organisms, and the removal or even desirability of removal of the plasmids would not be viewed by someone having ordinary skill in this art as being equivalent or even suggestive of each other. By their affirmative suggestion that their curing methods should be applicable to other types of plasmids in the *Agrobacterium* species, Uraji's own authors obviously are not suggesting to extrapolate their technique for use in curing plasmids in other bacterial species. Someone having ordinary skill in this art would likewise not have been reasonably expected Uraji's method to be useful for curing *E. coli* or other types of non-*Agrobacterium* species.

In the December 23, 2008 Office Action, the Examiner discounted Appellants' point that Uraji describes curing techniques only for *Agrobacterium* and not for *E. coli*, stating that there is no indication that the Uraji's methods would not work in other bacteria, such as *E. coli* (also a gram negative bacterium). Actually, it is respectfully noted that there is an indication that Uraji's methods would not be expected to apply to other bacteria. In their paper, the authors of Uraji et al. (who are at least persons having ordinary skill in the art or arguably even more skilled than the average biologist) note specifically that their curing technique "...should also be applicable to **other types of plasmids in *Agrobacterium* groups**..." (see abstract of Uraji et al.). *Agrobacterium* groups alone are stated as the bacteria for which the curing methods would be expected to work, and a fair reading would not extrapolate (reasonably) that Uraji et al.'s methods would be useful for curing plasmids in non-*Agrobacterium* bacterial species. It follows then that someone having ordinary skill in the art would not have reasonably expected

this curing method to be efficient in much more distantly related species such as *E. coli*, whether gram-negative or not. In fact, if it were the case that the authors believed their methods would be applicable for curing of bacteria from other genera, this most likely would have been stated in the paper, in order to increase the impact of their publication.

Further, Uraji teaches the introduction of the sacB gene on only one plasmid; the second plasmid does not have sacB gene. In the May 11, 2010 Office Action at page 6, the Examiner argues that "if two plasmids are being introduced in to the cell, it would follow that both plasmids would contain the sacB gene in order to cure the cell." This is an interpretation of the Uraji reference that may or may not be correct – and certainly Uraji itself provides no clues to knowing. With all due respect, the rejection is based on a fluctuating interpretation of what Uraji teaches – how can someone having ordinary skill be assumed to reach the same conclusion, AND be reasonably lead to apply that to the teachings of the other references.

In the October 19, 2010 Office Action at page 5, the Examiner argues that

> "Uraji does not teach the introduction of two sacB genes, it only teaches the introduction of one sacB gene. . . . Knowing that sacB was used successfully by Uraji to cure the first plasmid, there is a reasonable expectation of success that that second plasmid could be cured."

The introduction of two sacB genes into the pMut1 and pMut2 plasmids is an essential step to achieve the strain of claim 1. Uraji, or any of the other cited references, does not teach or suggest the deliberate and separate introduction of two sacB genes, one into each of the pMut1 and pMut2 plasmids. Again, there is no motivation to cure both plasmids from DSM 6601. Uraji as a primary reference falls short to help establish a prima facie case of obviousness.

The USPTO examination guidelines provide that, where an element from a secondary reference is substituted into the teaching from the primary reference, the primary reference should at least suggest that the modification could be successful (Exam Guideline B). Here, Uraji as a primary reference falls short to help establish a prima facie case of obviousness.

Regarding the **Blum-Oehler** reference, the Examiner cites it on the basis that it teaches that DSM 6601 is useful as a probiotic drug against intestinal disorders, and its method is a much safer way of curing, and further that Blum-Oehler states that the pMut1 and pMut2 plasmids are cryptic and have no apparent benefit to the host. Appellants agree that Blum-Oehler et al. describes the cryptic plasmids pMUT1 and pMUT2 of *E. coli* strain Nissle 1917 (syn. DSM 6601). In particular, this reference describes a PCR-based method for the specific detection of *E. coli* DSM 6601, which can be used to specifically detect *E. coli* DSM 6601 in the fecal flora of patients in the presence of other *E. coli* strains. The DNA sequences of the plasmids are described and their use for diagnostic and biotechnological purposes. The DSM 6601 strain of claim 1 is plasmid-free. At best, Blum-Oehler represents the problem in the art solved by the claimed plasmid-free strains – it presents absolutely no solution to its own problem.

As noted by the Examiner, Blum-Oehler does not suggest a plasmid-free DSM 6601 strain. The **Trevors** paper is relied upon for that, and Appellants believe that a fair reading of Trevors would not fill the gap left by Blum-Oehler. Even assuming arguendo that the desirability of having a plasmid-free *E. coli* strain DSM 6601 would be known, Trevors' mere description of several different ways to cure bacteria does not equate to successful curing of plasmids pMUT1 and pMUT2 from DSM 6601 such as in claim 1. In fact, the methods taught by Trevors to cure bacteria of plasmids would not be useful for the particular clone DSM 6601. Someone having ordinary skill in this art would not have found Trevors sufficiently instructive (and would not have been led) to effectively cure the DSM 6602 bacteria of plasmids pMUT1 and pMUT2 – such that it is "plasmid-free" as claimed.

Trevors et al. is a review article that describes several methods for "curing" bacteria from plasmids; however, none of these methods equate to the actual method the inventors used to construct the claimed plasmid-free strains – which method required significantly more than mere routine procedures (see page 2 of the instant application). Trevors is a general teaching at best, **even teaching against** a reasonable expectation for successful curing of plasmids in many circumstances:

> "[T]he usefulness of curing agents is unpredictable in many bacterial strains, as there are no standard protocols applicable to all plasmids" (p.149, c.1,

l.25).

"[M]any plasmids can not be cured..." (p.149, c.2, l.15).

"Although all of the plasmid curing agents discussed in this manuscript have been employed to enhance the frequency of plasmid lost, they are only useful against **some** plasmids. Therefore, when initially working with new plasmids and bacterial isolates a **wide variety of curing methods may have to be tried** prior to obtaining a satisfactory method." (emphasis added) (p.155, c.1, l.15).

Even if we were to assume that the desirability of having a plasmid-free *E. coli* strain DSM 6601 would have been known, does Trevors et al. make such a strain obvious? Would someone having ordinary skill in this art be motivated with a reasonable expectation of success to try each of the curing methods described in Trevors, especially when Trevors itself states how "unpredictable" the usefulness of these methods are, and "only useful against some plasmids", or even that "many plasmids can not be cured"? Appellants respectfully believe that the only reasonable answer must be "no." Trevors does not describe a predictable solution to the problem of obtaining a plasmid-free DSM 6601 strain. Trevors itself emphasizes the unpredictability of its own methods. This is underscored when Appellants' own specification is considered, in particular the details of the special paths taken to generate the claimed clones. (See the Examples section).

In fact, none of the methods described by Trevors equate to the actual method the inventors used to construct the claimed plasmid-free strains – which method required significantly more than mere routine procedures. As noted on page 2 of the subject application:

> It turned out in the exhaustive investigations that led to the present invention that plasmid-free clones of strain DSM 6601 cannot be prepared at all with normal genetic engineering methods or can be prepared only with great difficulty so that special paths must be taken in order to generate such clones. Since the wild type of the strain has two plasmids of different sizes in addition to its genomic DNA, the elimination of these plasmids must take place in several steps that take place in part in parallel.

The details of the special paths taken to generate the claimed strain are provided in the text of the instant application, especially in the Examples. Specific details about the challenges overcome, blind alleys pursued, and the like, are not provided, so at first glance it may appear as if eliminating the pMUT1 and pMUT2 of *E. coli* strain DSM

6601 was merely routine. This is simply not the case. The subject disclosure states that exhaustive investigations were undertaken to overcome the difficulties presented by elimination of the two plasmids of different sizes while preserving the genomic DNA of the E. coli.

Simply stated, Trevors is a general review article that teaches some procedures that are effective for curing some plasmids from some bacteria. A fair reading of it is that it discloses some choices for curing plasmids (e.g., different curing agents, antibiotics, and the like), any one of which could be selected for further research on Blum-Oehler's plasmid-containing DSM 6601. However, Trevors would not have been assessed by someone having ordinary skill in this art as providing a predictable solution to achieve a plasmid-free DSM 6601 using the routine measures it describes. As would be understood by someone having ordinary skill in this art, the general methodologies described by Trevors would not have been useful to remove the two plasmids in *E. coli* strain DSM 6601. Trevors is only background information, and does not provide the requisite motivation for modifying the Blum-Oehler strain.

The **Gasson** reference is cited for motivation to cure two or more plasmids, and even that reference deals with an entirely different strain (*Streptococcus*), and addresses a different problem, and solves its problem using a different curing process altogether. Gasson has been cited from a retrospective point of view – that is, to fill in a "gap" in the string of cited art, to show that a plasmid can be cured from a host. In Gasson's disclosure, a *Streptococcus* strain is cured of five plasmids.

Curing strains of *Streptococcus* having a large complement of plasmids requires several obstacles to be overcome. Difficulties addressed by Gasson include the assignment of suspected plasmid-encoded phenotypes to individual molecules, the complicated analysis of plasmid transfer experiments, and the equally complicated analysis of the molecular study of individual plasmids from the complement (as also noted by the Examiner in the Office Action dated May 11, 2010, at page 8). The focus of the Gasson article is how to deal with these particular issues. The curing process itself is conducted as a protoplastic-promoted curing, which means that plasmid-loss occurs during the early stages of protoplast regeneration.

In the situation of the *E. coli* DSM 6601 of the claimed invention, the pMut1 and pMut2 plasmids are both cryptic. There was no issue regarding which plasmid to cure. And of course, the method the inventors used for double-curing is completely different from the Gasson method. As is well accepted in this art – not every curing method is suitable for curing every bacterial species. The method employed by Gasson is nearly irrelevant to the method the inventors adapted, and would not have been effective to identify or address the problem encountered by the claimed method. The only parallel between the subject invention and Gasson is that a bacterial species is cured of more than one plasmid. However, the differences between the instant technology and Gasson's – different bacterial species, different problems encountered and overcome, different methods of curing – are simply too significant to be minimized or overlooked, which is what would be necessary to have this reference fill in the "gap" of the other cited art. For the purposes of this particular invention, Gasson would not have been a reference someone skilled in this art would have looked to for direction to cure DSM 6601 of the pMut1 and pMut2 plasmids.

In order to combine these references to make the current claims obvious, hindsight reconstruction is necessary using the applicants' own disclosure as a basis. The primary reference is too irrelevant, and the secondary references are too disconnected from the subject invention. It is impermissible to use hindsight reconstruction to pick and choose among isolated disclosures in the prior art to deprecate the claimed invention. (In re Fine, 837, F.2d 1071 (Fed. Cir. 1988); In re Fritch 972 F.2d 1260 (Fed. Cir. 1992). Using the inventor's success as evidence that one of ordinary skill in the art would have reasonably expected success represents an impermissible use of hindsight. Life Technologies, Inc. v. Clontech Laboratories, Inc., 224 F.3d 1320 (Fed. Cir. 2000).

To underscore the difficulties that were necessary for the inventors to overcome in achieving the claimed invention, attached is a copy of an article by Schultz et al. (2005), "Green fluorescent protein for detection of the probiotic microorganism Escherichia coli strain Nissle 1917 (EcN) in vivo", Journal of Microbiological Methods 61, 389-398. In this article, the original, plasmid-containing *E. coli* DSM 6601 strain is genetically manipulated to carry a foreign plasmid with the genetic information for the green

fluorescent protein. As shown and concluded therein, the foreign plasmid was not stable in vivo (see Abstract and the discussion beginning on page 395). Manipulation of DSM 6601/Nissle 1917 to alter its plasmid content had limited success in Schultz et al., even using a well-known marker gene *gfp* (green fluorescent protein) under the control of the *lac* promoter and following standard procedures for stable transformation (page 390-391). In response to Appellants' submission of this article, the Examiner in the December 23, 2008 Office Action, page 4, discounted Schultz as relevant on the grounds that it does not teach the curing of plasmids or the use of endogenous plasmids. However, on page 391, first column, lines 5-8, the authors do describe how the *E. coli* transformed with *gfp* (the EcN-GFP strain) was cured of the plasmid pUC-*gfp* used to transfer the *gfp* gene, and that this was done successfully. However, the resultant altered strain was not stable and ultimately rejected the plasmid. This was apparently a surprise to the authors, who expected the plasmid to be stable at least as long as selective conditions were maintained (see pages 396-397). We submit this paper to show that, even as late as 2005, the construction of a plasmid-free DSM 6601 derivative is not a routine or obvious matter.

In addition to the above points, it is noted that the strain of claim 1 has features and advantages that are neither taught nor suggested by the combination of four references. As stated in the subject application at page 4, first paragraph, one major advantage of the new strain is that, with the condition of the loss of the plasmids plus the fact that there was no change of the genomic DNA, the strain can be readily used as a cloning vehicle.

> Thus, they can be safely used in the laboratory as host cell for the cloning and expression of a plurality of genes and proteins. Experiments with strain DSM 6601 ΔpMut1/2 have shown that it is an especially good accepter for foreign DNA when the latter is integrated into its own plasmids present in isolated form, that is, therefore its own plasmids function as cloning vectors for the foreign DNA. Furthermore, since they are derived from a non-pathogenic strain, they can be used for the treatment of disturbances of the gastrointestinal tract in animals and humans. To this end they can be transformed, if desired with foreign genes that further the adhesion of the bacteria to the mucosa such as, e.g., adhesines that further the adhesion of the bacteria, optionally host-animal specifically, to the

mucosa of, e.g., cattle and/or swine and thus hinder or prevent the growth of other pathogenic microorganisms. (Specification at page 4).

It is submitted that claim 1 is non-obvious and patentable over the combination of Uraji et al. in view of Blum-Oehler et al (WO 99/44134) in view of Trevors et al., and Gasson.

2. Issue No. 2: Whether claims 2-6 are patentable under 35 U.S.C. §103(a) over Uraji et al. in view of Blum-Oehler et al (WO 99/44134) in view of Trevors et al., and Gasson, and further in view of Alexeyev et al. Appellant respectfully asserts that they are.

Main claim 2 includes the following steps for preparing a plasmid-free clone for DSM 6601:

a) introducing a resistance gene into plasmids pMut1 and pMut2,

b) introducing the *sacB* gene into the plasmids obtained in step a) so as to produce a pMut1 plasmid carrying a resistance gene and a *sacB* gene, and a pMut2 plasmid carrying a resistance gene and a *sacB* gene,

c) introducing the altered pMut1 and pMut2 plasmids obtained in step b) into the Escherichia coli strain DSM 6601, and cultivating the strain obtained thereby under conditions in which the naturally occurring pMut1 and pMut2 plasmids are displaced by the altered pMut1 and pMut2 plasmids obtained in step b); and

d) cultivating the clones obtained in step c) that substantially only permit the growth of bacteria that lack the *sacB* gene, so that the Escherichia coli containing the altered pMut1 and pMut2 plasmids do not grow, and thereby is produced a plasmid-free clone of Escherichia coli strain DSM 6601.

To find claims 2-6 obvious, the Examiner needed to cite a string of five references -- and as noted above, **no reference teaches the curing of DSM 6601 or the desire to cure it of both pMut1 and pMut2**. Regarding the five cited references, the comments above regarding four of them – Uraji et al., Blum-Oehler et al., Trevors et al. and Gasson et al.– are believed to be equally applicable here. That is, to summarize briefly:

(1) Uraji only describes the curing a non-*E. coli* strain (*Agrobacterium*) of a single plasmid via the introduction of the sacB gene; the second plasmid does not have sacB gene, and is not cured.

(2) Blum-Oehler represents the problem in the art solved by the claimed plasmid-free strains – it presents absolutely no solution to its own problem.

(3) Trevors is a general review article discussing known methods for "curing" bacteria from plasmids – none of which equate with the method the inventors discovered that was ultimately successful to actually eliminate the pMUT1 and pMUT2 plasmids and preserve the genomic DNA in DSM 6601. Trevors even teaches that curing methods can be unpredictable, or only useful for certain plasmids, and that many plasmids can not be cured at all. Appellants do not dispute that it is well known that some plasmids can be cured from some bacteria; however, the particular method of claims 2-6 is not suggested by Trevors.

(4) Gasson describes how multiple plasmids are cured from *Streptococcus*, via a protoplastic-promoted curing, where plasmid-loss occurs during the early stages of protoplast regeneration. Besides the fact that more than one plasmid is ultimately cured from the host, Gasson is nearly irrelevant to claims 2-6.

Regarding **Alexeyev et al.**, this paper is cited for its description of a tetracycline resistance cassette in a plasmid. The Examiner is of course correct that this reference describes the introduction of a tetracycline-resistant cassette. However, this is not described for *E. coli* strain DSM 6601. Also, the particular combination of steps leading to a plasmid-free DSM 6601 strain, as set forth in claims 2-6, is not mentioned at all. Alexeyev et al. list potential applications for their plasmids with antibiotic-resistance gene cassettes and omega elements (see page 65 in this publication):

> "Besides vector construction and in vitro deletion/insertion mutagenesis, constructs described here can be used:
> (i) for construction of other gene cassettes and omega elements;
> (ii) for mobilization of restriction sites;
> (iii) as a source of restriction sites instead of linkers".

There is no teaching that Alexeyev et al. used antibiotic-resistance gene cassettes for curing of plasmids. In light of this lack of relevant disclosure, someone having ordinary

skill in this art, seeking to cure DSM 6601 of plasmids, would not find this reference applicable with any reasonable expectation of success.

Please note that not one of steps a), b), c) or d) are actually suggested by any of the references, much less the combination of these specific steps. Regarding step a), no reference has been cited that describes introducing a resistance gene into both plasmids pMut1 and pMut2. For this step the Examiner points to Uraji, but this reference only describes introducing resistance into a single plasmid. Alexeyev describes the introduction of a tetracycline-resistant cassette in vector construction (Office Action dated May 11, 2010, page 8); however, this is not described for construction of *E. coli* strain DSM 6601, nor is there any use of antibiotic-resistance gene cassettes for curing of plasmids.

Regarding step b), the Examiner cites Uraji which, as explained above, only teaches the introduction of a single *sacB* gene into a single plasmid. Step b) requires the production of both a pMut1 plasmid carrying a resistance gene and a *sacB* gene, and a pMut2 plasmid carrying a resistance gene and a *sacB* gene.

Regarding step c), none of the three closest references – Uraji, Trevors or Gasson – describe how to transform Escherichia coli strain DSM 6601 with both the altered pMut1 and pMut2 plasmids, and cultivating the strain so that the naturally occurring pMut1 and pMut2 plasmids are displaced by the altered pMut1 and pMut2 plasmids. Uraji only deals with Agrobacterium and the removal of pTi plasmids, only one of which involves a *sacB*-gene altered plasmid. Trevors is cited by the Examiner to teach the general desirability to cure bacteria of plasmids (Office Action dated April 30, 2008, pages 3-4) – no particular method of Trevors is relied upon to teach this step or any step. Gasson is cited by the Examiner to generally teach that strains having more than one plasmid can be cured (Office Action dated May 11, 2010), although the Examiner acknowledges that Gasson's strain is *Streptococcus*, not *E. coli*, and the method used by Gasson is entirely different (protoplastic-promoted, where plasmid loss occurs during the early stages of protoplast regeneration).

Regarding step d), this is final step, of course, which produces a plasmid-free clone of Escherichia coli strain DSM 6601. None of the references describe double-

curing of the pMut1 and pMut2 plasmids, and therefore none can suggest or teach this step either.

Even assuming that one of the steps a) – d) are suggested, are all of the steps in combination obvious from these references? At best, each of the steps is seriously modified from what is taught by the respective reference cited by the Examiner. We submit that it is unreasonable to suppose that someone having ordinary skill in this art would have been lead to the combination of these method steps, using these five references, without the use of hindsight.

It is submitted that someone having ordinary skill in the art would not have found the method of claims 2-6 obvious with any or all of the five cited references in hand. The claimed method for curing *E. coli* strain DSM 6601 from both plasmids is not a technique that is merely a routine combination of the methods in the aforementioned publications which for an ordinary person skilled in the art would have found obvious.

Conclusion

For the above reasons, it is respectfully requested that these rejections be reversed.

viii. **Claims Appendix**

1. (Previously presented) A plasmid-free clone of Escherichia coli strain DSM 6601, which strain is identified as having accession number DSM 16700.

2. (Previously presented) The method of preparing a plasmid-free clone according to Claim 1, comprising the following steps:

a) introducing a resistance gene into plasmids pMut1 and pMut2,

b) introducing the *sacB* gene into the plasmids obtained in step a) so as to produce a pMut1 plasmid carrying a resistance gene and a *sacB* gene, and a pMut2 plasmid carrying a resistance gene and a *sacB* gene,

c) introducing the altered pMut1 and pMut2 plasmids obtained in step b) into the Escherichia coli strain DSM 6601, and cultivating the strain obtained thereby under conditions in which the naturally occurring pMut1 and pMut2 plasmids are displaced by the altered pMut1 and pMut2 plasmids obtained in step b); and

d) cultivating the clones obtained in step c) that substantially only permit the growth of bacteria that lack the *sacB* gene, so that the Escherichia coli containing the altered pMut1 and pMut2 plasmids do not grow, and thereby is produced a plasmid-free clone of Escherichia coli strain DSM 6601.

3. (Original) The method according to Claim 2, characterized in that the resistance genes are present in an expression cassette.

4. (Previously presented) The method according to Claim 2, characterized in that the resistance genes are selected under tetracycline resistance or kanamycin resistance.

5. (Previously presented) The method according to claim 2, characterized in that plasmid pMut1 is marked with a tetracycline resistance cassette and the *sacB* gene and that the original plasmid pMut2 is marked with a kanamycin resistance cassette and the *sacB* gene.

6. (Previously presented) The method according to claim 5, in which the bacteria transformed with plasmid pMut1, that is marked with a tetracycline resistance cassette and the *sacB* gene, are cultivated on plates containing tetracycline and subsequently on plates containing saccharose, and that after elimination of plasmid pMut1 in the first step elimination of plasmid pMut2 takes place by cultivation on kanamycin plates and further cultivation on saccharose plates.

7. (Canceled)

8. (Canceled)

ix. **Evidence Appendix**

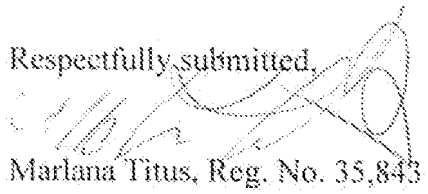
With the September 8, 2008 Amendment, Appellants submitted the article Schultz et al. (2005), "Green fluorescent protein for detection of the probiotic microorganism Escherichia coli strain Nissle 1917 (EcN) in vivo", Journal of Microbiological Methods 61, 389-398. The Examiner indicated this reference was entered in the record in the December 23, 2008 Office Action.

x. Related Proceedings Appendix

None

Accordingly, reversal of the rejection and allowance of the application are respectfully requested.

Respectfully submitted,



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Green fluorescent protein for detection of the probiotic microorganism *Escherichia coli* strain Nissle 1917 (EcN) in vivo

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Abstract

Probiotic microorganisms are defined as viable nutritional agents conferring benefit to the health of the human host. Especially, *Escherichia coli* strain Nissle 1917 (EcN) was shown to be equally effective as mesalazine in the maintenance of remission in ulcerative colitis (UC). Presumably, the therapeutic effect of EcN is linked to the presence of the strain in the region of interest; however, it remains difficult to follow the orally administered strain on its passage through the complex microbial environment of the intestine in vivo, inhabited dominantly by various *E. coli* strains, using traditional culturing methods. In this study we transformed EcN and a wild-type *E. coli* from a laboratory rat (EcR) with a plasmid carrying a *gfp* gene (pUC-*gfp*) to obtain EcN- and EcR-GFP to allow in vivo detection without alteration of strain-specific characteristics. Analysis of different strain-specific characteristics included the measurement of stimulation of IL-8 secretion and adhesion in vitro using the epithelial cell line HT-29. The kinetics of intestinal distribution in mice and colonization properties in rats following oral administration was studied in vivo. Detectability of the strain in histologic specimens was analysed using fluorescence microscopy and immunohistochemistry. The identity of fluorescent *E. coli* strains isolated from stool samples, Peyer's patches (PP) and mesenteric lymph nodes (MLN) was determined by REP-PCR. We were able to demonstrate that EcN and EcN-GFP do not differ in stimulation of IL-8 secretion or adhesion to HT-29 cells. In vivo, EcN-GFP colonies were readily detectable by fluorescence microscopy in luminal samples and also by immunohistochemistry in histological sections allowing analysis of the kinetics of the intestinal passage following oral administration. Translocation of fluorescent and non-fluorescent bacteria into PP and MLN was noted at 6 h post oral administration. EcN-GFP was detectable initially for 14 days in faecal samples of rats, while EcR-GFP was detectable throughout the whole experiment (45 days). Challenge with ampicillin at day 45 demonstrated continuing presence of EcN-GFP in small numbers by

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reappearing fluorescent colonies. The plasmid was not stable in vivo since non-fluorescent EcN colonies were detected also in faecal samples by REP-PCR.

In summary, transformation of EcN to obtain EcN-GFP in our study had no detectable influence on the probiotic microorganism regarding adhesion on and induction of IL-8 secretion of HT-29 cells and allows the detection in mixed microbial environments in vivo but the stability of EcN-GFP in vivo is limited.

Keywords: Probiotics; Green fluorescent protein; *E. coli* Nissle 1917

1. Introduction

Probiotic microorganisms, such as different Lactobacilli, Bifidobacteria, and Enterobacteriaceae, are defined as viable nutritional agents conferring benefit to the health of the human host (Lilly and Stilwell, 1965). Clinically, beneficial effects of probiotics have been demonstrated for the treatment of infectious diarrhea in infants (Isolauri, 2003), amelioration of side-effects of antibiotic therapy (Cremonini et al., 2002), prevention of allergies (Bienenstock et al., 2002), and treatment of inflammatory bowel diseases (IBD) (Schultz et al., 2003a,b). Especially, *Escherichia coli* strain Nissle 1917 (EcN) was shown to be equally effective as mesalazine in the maintenance of remission in ulcerative colitis (UC) (Kruis et al., 1997, 2004; Rembacken et al., 1999).

The detailed mechanisms by which probiotic bacteria mediate their effects are beginning to be elucidated (Rachmilewitz et al., 2004) but are still not fully understood and seem complex and *diverse* among different probiotic preparations (Bai et al., 2004; Ulisse et al., 2001). Studies in humans, animals, and in vitro suggest that probiotic bacteria modulate the composition of the intestinal microflora (Tannock et al., 2000) or enhance the intestinal mucosal barrier (Mack et al., 1999; Madsen et al., 2001). A direct, anti-inflammatory influence on the intestinal immune system has also been suggested (Rachmilewitz et al., 2004; Malin et al., 1996; Schultz et al., 2003a,b).

Further in vitro experiments have suggested that probiotic bacteria are able to immunomodulate the cytokine response of intestinal mucosal cells in response to luminal antigens (Borruel et al., 2003), but in vivo confirmation with regard to the complex relationships involving possible interspecies and intraspecies interactions with orally administered probiotic bacteria are rare (Boudeau et al., 2003; Mack et al., 1999).

Presumably, the therapeutic effect of a probiotic organism can be linked to its presence in the region of interest. Especially with the probiotic EcN it remains difficult to follow the orally administered strain on its passage through the complex microbial environment of the intestine in vivo, inhabited dominantly by different *E. coli* strains, using traditional culturing methods (Simon and Gorbach, 1984).

For the present report, we transformed EcN with a high-copy number plasmid carrying a modified *gfp* gene to obtain EcN-GFP, suitable for in vivo detection in mixed cultures or in histological sections. We further analysed the impact of labelling on strain-specific properties in vivo and in vitro and addressed questions regarding colonization kinetics and host cell interaction.

2. Methods and materials

2.1. Experiments in vitro

2.1.1. Bacterial strains and transformation

EcN was obtained from Ardeypharm GmbH, Herdecke, Germany. A wild-type strain of *E. coli* (EcR) was cultured from the faeces of a laboratory rat. The isolates were maintained on Columbia blood agar (CBA) and MacConkey agar plates (Merck, Darmstadt, Germany). Transformation of EcN and EcR to obtain EcN-GFP and EcR-GFP with plasmid pUC-*gfp* was performed using standard techniques (Sambrook et al., 1989). In brief, pUC-*gfp* was derived from the high-copy number vector pUC18 by insertion of a modified *gfp* gene under control of the *lac* repressor, allowing single cell detection by fluorescence microscopy (Crameri et al., 1996). Bacterial cells harbouring the plasmid were maintained on Mueller–Hinton-agar (Merck) or in Luria–Bertani broth (LB; Merck) in the presence of 100 mg/l ampicillin (Ratiopharm, Ulm, Germany). Fluorescence was

detected using an UV lamp (Micro-Bio-Tec, Gießen, Germany; 365 nm, 2×4 W) or a fluorescence microscope (Leica DMR X, Leitz, Wetzlar, Germany; fluorescence lamp Osram HBO 100 W, excitation filter 450–490 nm, band-elimination filter 515–560 nm). EcN-GFP was cured from the plasmid (EcN-cGFP) by repeated subculture to antibiotic-free media until disappearance of fluorescence and loss of resistance to ampicillin.

2.1.2. Stimulation of HT-29 cells and determination of interleukin (IL)-8 secretion

HT-29 intestinal epithelial cells at a concentration of 200,000/well of a 24 well plate were incubated at 37 °C in room air supplemented with 10% CO_2 for 24 h in antibiotic-free Dulbecco's MEM medium (GIBCO/BRL Invitrogen, Karlsruhe, Germany, 1 g/l D-glucose; 3.7 g/l $NaHCO_3$; 1.02 g/l *N*-acetyl-L-alanyl-L-glutamine) supplemented with 10% fetal calf serum (FCS; PAN Biotech, Aidenbach, Germany), non-essential amino acids, and 1% Na-Pyruvate (Biochrom, Berlin, Germany). Following renewal of the medium, co-incubation of EcN or EcN-GFP at concentrations from 1×10^2–1×10^{10} CFU/ml and HT-29 cells was performed for 16 h, and IL-8 was determined in the supernatant by ELISA (EH2-IL-8; Endogen, Perbio Science, Bonn, Germany). Viability of the cells was determined by trypan blue exclusion. As positive control, stimulation of the cells with 125 μg TNF-α/well (R and D Systems, Wiesbaden-Nordenstadt, Germany) was performed. All experiments were performed in duplicate and repeated three times.

2.1.3. Adhesion assay

To test for the influence of the labelling of EcN-GFP on adhesion to HT-29 intestinal epithelial cells, a standard adhesion assay was used (Boudeau et al., 2003). Bacteria (EcN, EcN-GFP, *E. coli* HB101 (as control)) were cultivated in liquid LB broth over night at 37 °C without agitation. Of a dilution (A_{600}=0.01) from the overnight culture, 8 μl were used to infect a confluent HT-29 monolayer in a well of a microtiter plate. Confluency of the HT-29 cell monolayer was reached after 24 h incubation under cell culture conditions. The infected HT-29 cells were incubated under cell culture conditions for 2 h. Each well was subsequently washed with 100 μl EBSS (Earl's balanced salt solution) three times. Addition of 100 μl Triton X-100 and shaking for 20 min resulted in lysis of the epithelial cells. Appropriate dilutions of the lysate were plated on LB agar plates. The number of HT-29 associated CFU was counted (Oelschlaeger and Tall, 1997).

All experiments were repeated at least three times and three wells per condition were used.

2.2. Experiments in vivo

2.2.1. Intestinal distribution and kinetics of EcN-GFP in mice following oral administration

Commercially available Balb/C mice at 8–12 weeks and 18–20 g body weight (Charles River, Sulzfeld, Germany) were orally inoculated with a single-dose of 200 μl sterile saline containing 5×10^{10} CFU/ml EcN-GFP. At 1, 3, 6, 12, 24, 48, and 72 h post gastric lavage, two animals and one control animal were sacrificed by CO_2 asphyxation. Liver, spleen and all detectable MLN and PP were removed aseptically before the intestine was opened. Cardiac blood was taken and used undiluted. To minimize the risk of contamination, the intestinum was opened at the end of the procedures to take samples from luminal contents from the stomach, cecum, and rectum. All samples were weighed, homogenized, serially diluted in saline, and cultured on CBA and MacConkey plates (Merck, Darmstadt) overnight at 37 °C in air supplemented with 5% CO_2. Numbers of CFU were normalized by weight.

2.2.2. Immunohistochemistry for EcN-GFP

Intestinal tissue was washed vigorously in sterile PBS, embedded (Tissue Tek® O.C.T. compound 4583; Zoeterwoude, The Netherlands), and frozen in liquid nitrogen using an *iso*-pentane heat buffer to prevent snap-freezing. Cryosections of intestinal tissue were first blocked with FCS and then incubated in PBS containing anti-GFP rabbit polyclonal IgG antibody. As secondary antibody, an anti-rabbit IgG-HRP was used (Santa Cruz Biotechnology, Santa Cruz, CA), according to the manufacturer's instructions of the VECTOR®NovaRED substrate kit (Vector Laboratories, Burlingame, CA).

2.2.3. Colonization of rats and intermittent antibiotic challenge

Commercially available Fisher rats (Charles River) (n=4) were treated with ampicillin (50 mg/kg body weight) in drinking water for 3 days and then challenged on three consecutive days by gastric lavage with 1000 μl PBS containing 3–4×10^9 CFU of EcN-GFP or EcR-GFP. Stool samples (1 g wet weight) were collected on days 0, 3, 7, 10, 14, 17, 23, 30, 37, and 45, diluted and homogenized in 100 μl sterile saline. Serial dilutions were cultured on blood agar plates at aerobic conditions. After 24 h, plates were inspected under UV light. As soon as the absence of fluorescent colonies from faecal samples was noted (day 14 and day 37), rats were given ampicillin (50 mg/kg body weight) (days 15–17 and days 38–40) or clindamycin (50 mg/kg body weight; days 31–33) in drinking water, followed by culture of the faecal flora.

2.2.4. Typing of E. coli isolates by repetitive extragenic palindromic PCR (REP-PCR)

REP-PCR was performed according to Versalovic et al. (1991). Briefly, after adjustment of the concentration of genomic DNA, PCR was performed using primers REP-1 5′-GCGCCGICATCAGGC-3′ and REP-2 5′-ACGTCT-TATCAGGCCTAC-3′ with 30 cycles of 94 °C for 1 min, 40 °C for 1 min and 65 °C for 8 min. Patterns with different bands were considered to represent different *E. coli* strains.

2.2.5. Detection of EcN-GFP by fluorescence microscopy

Tissue sections from the stomach, jejunum, ileum, caecum, colon, and rectum were embedded (Tissue Tek®) and frozen in liquid nitrogen using an *iso*-pentane heat buffer to prevent snap-freezing and tissue damage. Frozen samples were stored at −70 °C until further use. Frozen sections (3–4 μm thick) and H+E stained sections were examined using native contrast and fluorescent light. Images were taken with a digital camera (RT Color Spot 2.2.1., Diagnostic Instruments Inc., St. Sterling Heights, MI).

2.3. Statistical analysis

For statistical analysis, Sigma Stat 2.03 (SPSS Inc., Chicago, IL 60606 USA) software was used. To control for normally distributed variables, the Kolmogorow–Smirnow test was used, followed by a *t*-test. A $p<0.05$ was regarded as significant, while higher values were regarded as non-significant (ns).

3. Results

3.1. Experiments in vitro

3.1.1. Stability of EcN-GFP in vitro

Transformation of EcR and EcN with plasmid pUC-*gfp* resulted in clones EcR-GFP and EcN-GFP with uniform and bright display of fluorescence of single colonies after 24 h on LB agar plates containing 100 mg/l ampicillin. Daily serial subculture of EcN-GFP on Mueller–Hinton-agar without ampicillin demonstrated that after 14 days more than 50% of approximately 1000 CFUs harboured the plasmid, as evidenced from fluorescence. Subcultures of non-fluorescent colonies failed to grow on Mueller–Hinton-agar supplemented with ampicillin. Under the microscope at ×1000 magnification and fluorescent light, single fluorescent bacterial cells with different intensity of fluorescence could be detected (data not shown).

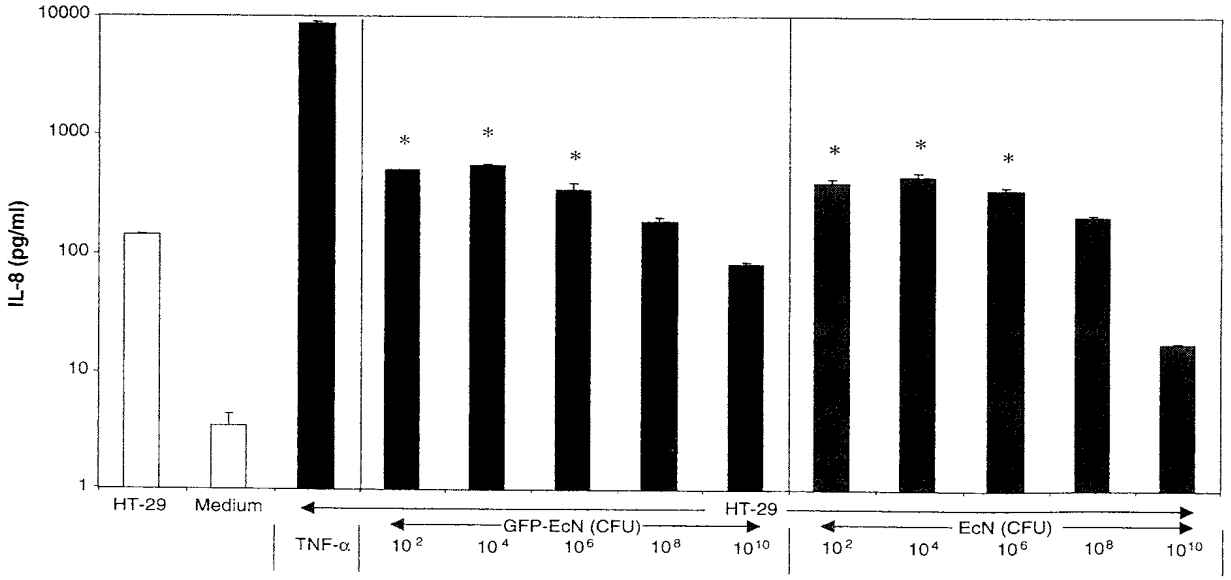

Fig. 1. Comparison of the ability of EcN and EcN-GFP to stimulate the secretion of IL-8 in HT-29 epithelial cells. There was no significant difference between the original EcN and EcN-GFP. *$p\leq0.05$ vs. unstimulated HT-29 cells. Shown is one of at least three experiments.

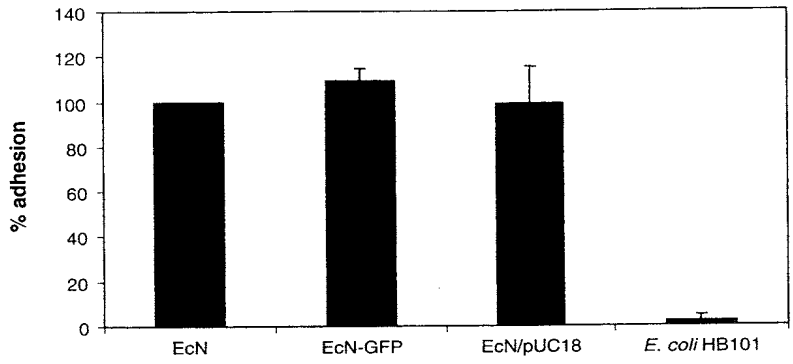

Fig. 2. Adhesion of different bacterial strains to HT-29 epithelial cells. No difference in adherence efficiency was observed with EcN-GFP and EcN containing the empty plasmid vector compared with EcN. *E. coli* HB 101, with no adhesion to epithelial cells, was used as negative control.

3.1.2. In vitro stimulation of IL-8 secretion by HT-29 cells

Secretion of IL-8 from HT-29 cells was stimulated by co-incubation with EcN and EcN-GFP in vitro. IL-8 secretion was dose-dependent with maximum values observed at concentrations of 10^2 to 10^4 CFU/ml. No significant difference in secretion of IL-8 could be demonstrated for stimulation with EcN or EcN-GFP (Fig. 1).

3.1.3. In vitro adherence to HT-29 cells

Adherence to host epithelial tissue was investigated in vitro by co-incubating EcN and EcN-GFP with HT-29 cells for 1–3 h. No alteration of in vitro adhesion of EcN-GFP in comparison to EcN to HT-29 cells was noted (Fig. 2).

3.2. Experiments in vivo

3.2.1. Intestinal passage and localisation of EcN-GFP in vivo

We analysed the kinetics of the intestinal passage of EcN-GFP following oral administration in more detail in Balb/c mice. After a single dose of EcN-GFP, fluorescent colonies were detectable in subcultures of faecal samples on CBA plates (Fig. 3). One hour after ingestion, EcN-GFP was detectable in high numbers in the stomach and in lower numbers in the caecum, but

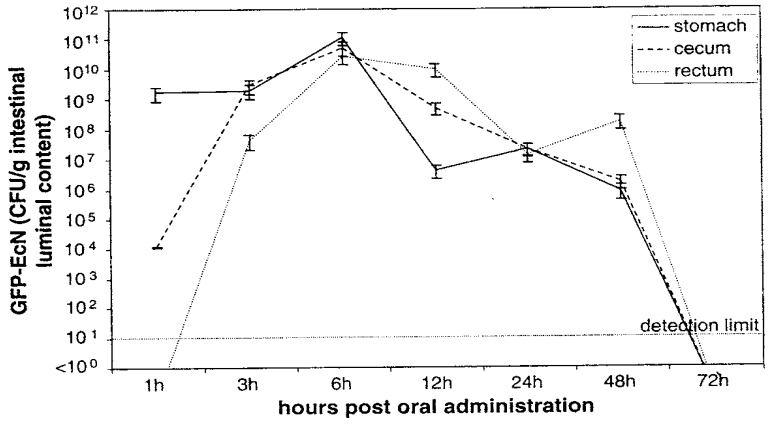

Fig. 3. Intestinal passage of EcN-GFP following single oral administration. EcN-GFP was readily detectable in intestinal luminal content, cultured on CBA up to 48 h.

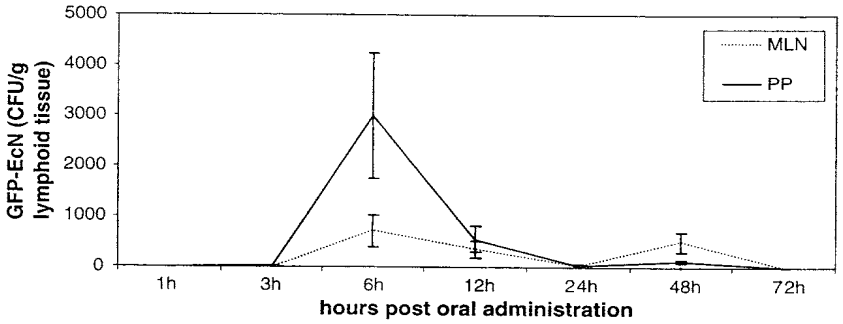

Fig. 4. Bacterial translocation of EcN-GFP to mesenteric lymph nodes (MLN) and Peyer's patches (PP). Detection of fluorescent colonies following homogenization of lymphoid tissue and subculturing on CBA. No fluorescent colonies were detected in blood or splenic tissue.

not in the rectum. At 6 h post administration the microorganism was equally present throughout the intestine (stomach, cecum, and rectum) with maximum concentrations of approximately 5×10^{10} CFU/g faeces in all intestinal sections as determined by subculture. At this time-point, viable microorganisms (*e.g. enterococci, E. coli* with and without fluorescence) were subcultured for the first time from at least three homogenized PP and MLN from different locations of the intestine (Fig. 4). No organisms were detected in the spleen, liver or blood of the animals at all time-points. By fluorescent microscopy and immunohistochemistry, it became apparent that the organisms were mainly localized close to the epithelial surface of the stomach, cecum, and rectum and also in the lumen of the intestine (Figs. 5 and 6).

3.2.2. Colonization of rats and intermittent antibiotic challenge

The colonization of Fisher rats by EcN in comparison to EcR was monitored after a 3-day oral antibiotic pre-treatment with ampicillin followed by a 3-day period of oral administration of EcN-GFP or EcR-GFP. Following oral administration of $3–4\times10^{9}$ CFU the numbers of EcN-GFP in faeces steadily declined, until at day 14 faecal cultures for EcN-GFP were negative. In contrast, the numbers of EcR-GFP remained stable. Following renewed administration of ampicillin on days 15–17, concentrations of EcN-GFP and also EcR-GFP increased to reach a maximum of 1×10^{11} CFU/g on day 17. Again, numbers of EcN-GFP declined with time and EcN-GFP was not detectable at day 45, despite administration of

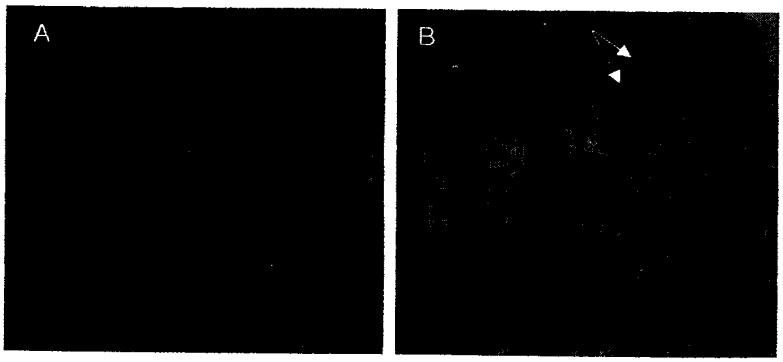

Fig. 5. Fluorescence microscopy of histological sections of the caecum from untreated controls (A, autofluorescence of intestinal tissues) and from mice challenged with EcN-GFP (B). EcN-GFP appears as a bright band close to the epithelial surface on the bottom of the crypt (arrows)(×400).

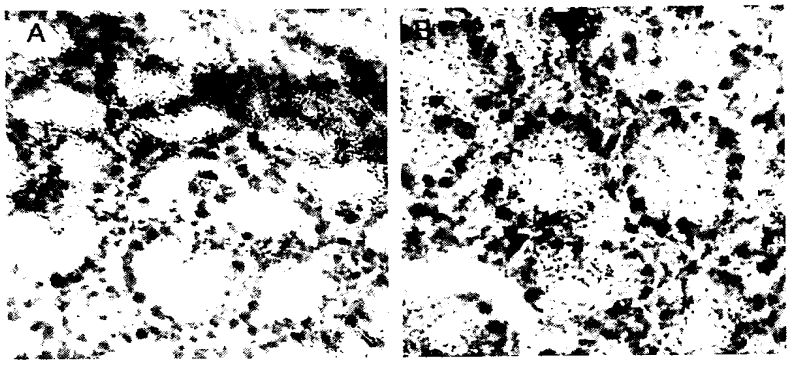

Fig. 6. Immunohistochemical analysis of the caecum of an untreated mouse (A) and an animal, challenged with EcN-GFP (B). EcN-GFP appears red–brown on the luminal surface of the epithelium. The section is counterstained with H and E (×400).

clindamycin at days 31–33 or ampicillin at days 38–40. EcR-GFP concentrations remained stable up to day 45 (Fig. 7).

3.2.3. Stability of pUC-gfp as a marker in vivo

REP-PCR of EcN-GFP revealed a pattern distinct from patterns from non-fluorescent *E. coli* cultured from the faeces of rats prior to feeding (data not shown). After feeding, all colonies with fluorescence demonstrated the pattern of EcN-GFP, however, non-fluorescent colonies with the pattern of EcN were found (data not shown).

4. Discussion

Probiotic microorganisms are defined as viable nutritional agents conferring benefits to the health of the human host (Lilly and Stilwell, 1965). Despite mounting evidence for clinical benefits of EcN (Schultz et al., 2003a,b), the mechanisms by which this organism mediates its anti-inflammatory effects are just beginning to be elucidated but still are not well understood (Rachmilewitz et al., 2004). In the case of EcN, this is largely due to technical difficulties since it remains a challenge to study the fate of this micro-organism, administered orally, in a complex microbial environment in vivo such as the *human* intestinal bowel flora, in which numerous *E. coli* strains represent the dominant enterobacterial species (Adlerberth et al., 1998; Kühn et al., 1986; Nowrouzian et al., 2003). Furthermore, it has been shown that the faecal flora does not represent the whole diversity of the intestinal microbial environment. Especially in IBD, a different composition of the microflora originated from healthy and diseased sections of the gut were seen close to or within the mucosal layer (Swidsinski et al., 2002; Seksik et al., 2003).

Labelling of EcN with GFP enabled us to follow the intestinal passage of the orally administered strain EcN-GFP in mice to document bacterial translocation in PP and MLN and to compare colonization properties of EcN with that of an *E. coli* strain isolated from rat faeces, in vivo.

For that purpose, *gfp* under the control of the *lac* promoter was subcloned into the high-copy plasmid pUC18 to obtain high-level expression of GFP, necessary for single-cell detection at higher magnification in mixed cultures. However, since expression of GFP to high intracellular concentrations may influence main functions of the host strain, we compared EcN-GFP with the unlabelled strain in in vitro assays. EcN is known to stimulate NF-κB-dependent IL-8 secretion in HT-29 cells (Lammers et al., 2002). In our experiments, plasmid-carriage by EcN did not influence the dose-dependent stimulation of IL-8 secretion in HT-29 cells. Adhesion of EcN and EcN-GFP to HT-29 cells was studied, using a standard adhesion assay. Labelling of EcN with GFP did not alter the adhesion properties in vitro to HT-29 cells.

To link the effect of a probiotic organism to its presence in the region of interest, we analysed the use of EcN-GFP on a cellular level in a mouse model. Following oral administration of a single dose without

prior antibiotic treatment, the passage of EcN-GFP was monitored by fluorescent microscopy and immunohistochemistry of histological sections and by culture of luminal content on CBA. Due to the high expression of GFP, EcN-GFP was readily detectable close to the intestinal epithelial cell layer by fluorescence microscopy (Fig. 6) as well as in luminal contents by subculturing on CBA. Immunohistochemical analysis confirmed the location of EcN-GFP (Fig. 6). Bacterial translocation from the gastrointestinal tract of mice into organs of the intestinal immune system occurs on a regular basis (Berg, 1983; Wells et al., 1987). However, for the first time, translocation into PP and MLN of an orally administered viable probiotic microorganism was documented. This finding opens the possibility that probiotic microorganisms mediate their immunomodulatory effects through a direct contact with immunocompetent cells of the intestinal immune system (Rachmilewitz et al., 2004; Schultz et al., 2003a,b).

Plasmidic carriage of a marker gene may likely cause its loss at growth conditions without selective pressure. We demonstrated that pUC-*gfp* was detectable in clones of EcN after subculturing at non-selective conditions in vitro after 14 days. At the same time, as expected, clones with concomitant loss of fluorescence and resistance to ampicillin were observed, suggesting the loss of the plasmid. To apply the transformed EcN in vivo and study stability and detectability of the plasmid as well as colonization in comparison to an *E. coli* strain, isolated from rat faeces, we investigated the modified strain in a rat model. While EcN-GFP was detectable for up to 14 days following administration, the rat-derived EcR remained at a stable concentration throughout the whole experiment (day 45), most likely due to better adaptation and adhesion mechanisms. After direct selection for EcN-GFP by the administration of ampicillin, EcN-GFP was again detectable and re-grew to high numbers in the faeces, but numbers again declined until day 45. Re-growth of EcN-GFP was not recorded after the administration of clindamycin. The true number of EcN-GFP in faeces is probably underestimated due to the loss of

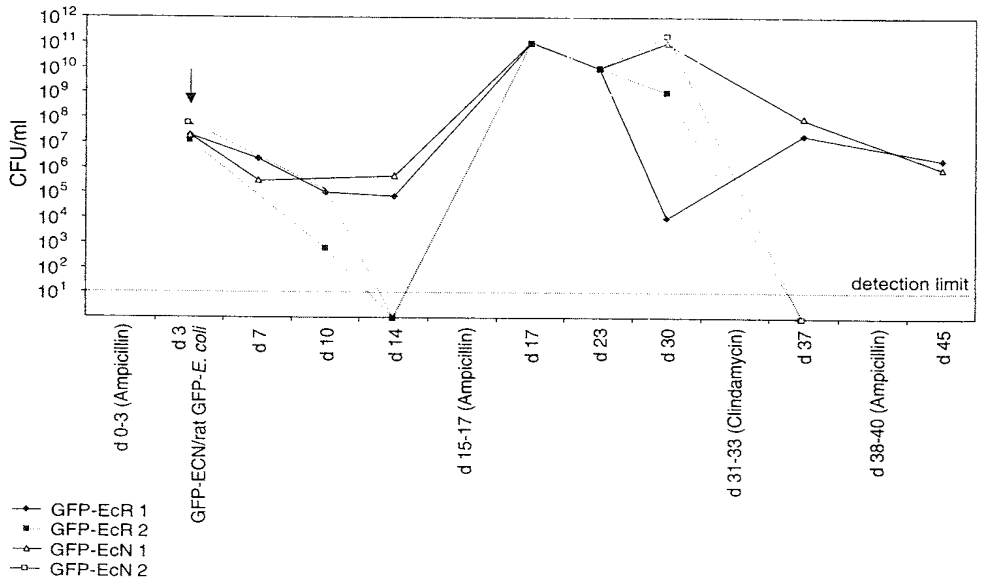

Fig. 7. Concentration of fluorescent colonies in faecal samples of rats, following a single oral challenge with a specific rat-derived *E. coli* (EcR-GFP ■/◆) and EcN-GFP (□/△) to study the colonization properties and the influence of antibiotics on the colonization process. A single dose of EcN-GFP and EcR-GFP were administered orally following three days of oral ampicillin. EcN-GFP was initially detectable in luminal samples for 14 days and again after oral antibiotic treatment with ampicillin up to day 37. The concentration of the rat-derived EcR was stable throughout the experiment until day 45. Administration of clindamycin on days 31–33 did not influence the luminal concentration of either EcN-GFP or EcR-GFP.

plasmid as demonstrated by REP-PCR. Faeces might also not truly represent the bacterial spectrum at different locations of the gut. Apart from GFP expression in vivo being not stable due to the lack of selection pressure and loss of plasmid, the possibility remained that the plasmid was acquired by other strains of the resident bowel flora. However, as documented by REP-PCR analysis from individual *E. coli* clones from faeces, all fluorescent and also numerous non-fluorescent colonies exhibited patterns undistinguishable from EcN. This indicates that pUC-*gfp* was not acquired by other enterobacteriaceae. However, anaerobic bacteria were not analysed.

In summary, GFP expressed by the probiotic *E. coli* strain Nissle 1917 from a high-copy plasmid allowed for the first time a study on the intestinal passage of an orally administered probiotic microorganism. In agreement with our in vitro observations, the strain adheres to the intestinal epithelial layer and immunomodulatory effects might be mediated by direct contact with immunocompetent cells of the host following translocation of viable microorganisms into PP and MLN. A limitation to the use of EcN-GFP is the instability of the marker in vivo due to the lack of applied selection pressure. This leads to underestimation of the concentration in faecal subcultures. Acquisition of the plasmid by other microorganisms was not observed. However, the use of EcN-GFP opens an array of experimental possibilities like the study of microbial–host interactions on the cellular level, the influence of the resident microflora, and concurrent antimicrobial treatment on colonization.

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plasmid as demonstrated by REP-PCR. Faeces might also not truly represent the bacterial spectrum at different locations of the gut. Apart from GFP expression in vivo being not stable due to the lack of selection pressure and loss of plasmid, the possibility remained that the plasmid was acquired by other strains of the resident bowel flora. However, as documented by REP-PCR analysis from individual *E. coli* clones from faeces, all fluorescent and also numerous non-fluorescent colonies exhibited patterns undistinguishable from EcN. This indicates that pUC-*gfp* was not acquired by other enterobacteriaceae. However, anaerobic bacteria were not analysed.

In summary, GFP expressed by the probiotic *E. coli* strain Nissle 1917 from a high-copy plasmid allowed for the first time a study on the intestinal passage of an orally administered probiotic microorganism. In agreement with our in vitro observations, the strain adheres to the intestinal epithelial layer and immunomodulatory effects might be mediated by direct contact with immunocompetent cells of the host following translocation of viable microorganisms into PP and MLN. A limitation to the use of EcN-GFP is the instability of the marker in vivo due to the lack of applied selection pressure. This leads to underestimation of the concentration in faecal subcultures. Acquisition of the plasmid by other microorganisms was not observed. However, the use of EcN-GFP opens an array of experimental possibilities like the study of microbial–host interactions on the cellular level, the influence of the resident microflora, and concurrent antimicrobial treatment on colonization.

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prior antibiotic treatment, the passage of EcN-GFP was monitored by fluorescent microscopy and immunohistochemistry of histological sections and by culture of luminal content on CBA. Due to the high expression of GFP, EcN-GFP was readily detectable close to the intestinal epithelial cell layer by fluorescence microscopy (Fig. 6) as well as in luminal contents by subculturing on CBA. Immunohistochemical analysis confirmed the location of EcN-GFP (Fig. 6). Bacterial translocation from the gastrointestinal tract of mice into organs of the intestinal immune system occurs on a regular basis (Berg, 1983; Wells et al., 1987). However, for the first time, translocation into PP and MLN of an orally administered viable probiotic microorganism was documented. This finding opens the possibility that probiotic microorganisms mediate their immunomodulatory effects through a direct contact with immunocompetent cells of the intestinal immune system (Rachmilewitz et al., 2004; Schultz et al., 2003a,b).

Plasmidic carriage of a marker gene may likely cause its loss at growth conditions without selective pressure. We demonstrated that pUC-*gfp* was detectable in clones of EcN after subculturing at non-selective conditions in vitro after 14 days. At the same time, as expected, clones with concomitant loss of fluorescence and resistance to ampicillin were observed, suggesting the loss of the plasmid. To apply the transformed EcN in vivo and study stability and detectability of the plasmid as well as colonization in comparison to an *E. coli* strain, isolated from rat faeces, we investigated the modified strain in a rat model. While EcN-GFP was detectable for up to 14 days following administration, the rat-derived EcR remained at a stable concentration throughout the whole experiment (day 45), most likely due to better adaptation and adhesion mechanisms. After direct selection for EcN-GFP by the administration of ampicillin, EcN-GFP was again detectable and re-grew to high numbers in the faeces, but numbers again declined until day 45. Re-growth of EcN-GFP was not recorded after the administration of clindamycin. The true number of EcN-GFP in faeces is probably underestimated due to the loss of

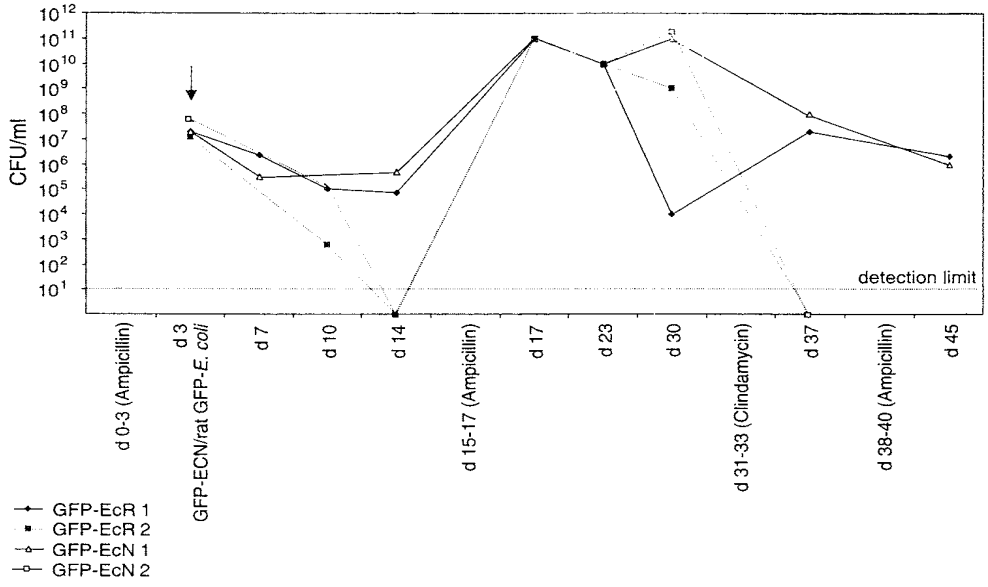

Fig. 7. Concentration of fluorescent colonies in faecal samples of rats, following a single oral challenge with a specific rat-derived *E. coli* (EcR-GFP ■/◆) and EcN-GFP (□/△) to study the colonization properties and the influence of antibiotics on the colonization process. A single dose of EcN-GFP and EcR-GFP were administered orally following three days of oral ampicillin. EcN-GFP was initially detectable in luminal samples for 14 days and again after oral antibiotic treatment with ampicillin up to day 37. The concentration of the rat-derived EcR was stable throughout the experiment until day 45. Administration of clindamycin on days 31–33 did not influence the luminal concentration of either EcN-GFP or EcR-GFP.

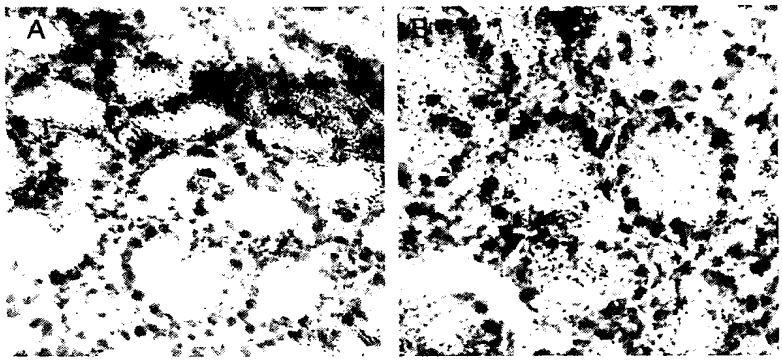

Fig. 6. Immunohistochemical analysis of the caecum of an untreated mouse (A) and an animal, challenged with EcN-GFP (B). EcN-GFP appears red–brown on the luminal surface of the epithelium. The section is counterstained with H and E (×400).

clindamycin at days 31–33 or ampicillin at days 38–40. EcR-GFP concentrations remained stable up to day 45 (Fig. 7).

3.2.3. Stability of pUC-gfp as a marker in vivo

REP-PCR of EcN-GFP revealed a pattern distinct from patterns from non-fluorescent *E. coli* cultured from the faeces of rats prior to feeding (data not shown). After feeding, all colonies with fluorescence demonstrated the pattern of EcN-GFP, however, non-fluorescent colonies with the pattern of EcN were found (data not shown).

4. Discussion

Probiotic microorganisms are defined as viable nutritional agents conferring benefits to the health of the human host (Lilly and Stilwell, 1965). Despite mounting evidence for clinical benefits of EcN (Schultz et al., 2003a,b), the mechanisms by which this organism mediates its anti-inflammatory effects are just beginning to be elucidated but still are not well understood (Rachmilewitz et al., 2004). In the case of EcN, this is largely due to technical difficulties since it remains a challenge to study the fate of this micro-organism, administered orally, in a complex microbial environment in vivo such as the *human* intestinal bowel flora, in which numerous *E. coli* strains represent the dominant enterobacterial species (Adlerberth et al., 1998; Kühn et al., 1986; Nowrouzian et al., 2003). Furthermore, it has been shown that the faecal flora does not represent the whole diversity of the intestinal microbial environment. Especially in IBD, a different composition of the microflora originated from healthy and diseased sections of the gut were seen close to or within the mucosal layer (Swidsinski et al., 2002; Seksik et al., 2003).

Labelling of EcN with GFP enabled us to follow the intestinal passage of the orally administered strain EcN-GFP in mice to document bacterial translocation in PP and MLN and to compare colonization properties of EcN with that of an *E. coli* strain isolated from rat faeces, in vivo.

For that purpose, *gfp* under the control of the *lac* promoter was subcloned into the high-copy plasmid pUC18 to obtain high-level expression of GFP, necessary for single-cell detection at higher magnification in mixed cultures. However, since expression of GFP to high intracellular concentrations may influence main functions of the host strain, we compared EcN-GFP with the unlabelled strain in in vitro assays. EcN is known to stimulate NF-κB-dependent IL-8 secretion in HT-29 cells (Lammers et al., 2002). In our experiments, plasmid-carriage by EcN did not influence the dose-dependent stimulation of IL-8 secretion in HT-29 cells. Adhesion of EcN and EcN-GFP to HT-29 cells was studied, using a standard adhesion assay. Labelling of EcN with GFP did not alter the adhesion properties in vitro to HT-29 cells.

To link the effect of a probiotic organism to its presence in the region of interest, we analysed the use of EcN-GFP on a cellular level in a mouse model. Following oral administration of a single dose without

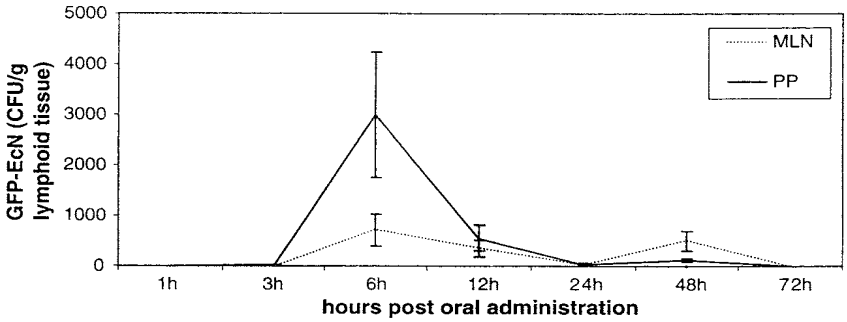

Fig. 4. Bacterial translocation of EcN-GFP to mesenteric lymph nodes (MLN) and Peyer's patches (PP). Detection of fluorescent colonies following homogenization of lymphoid tissue and subculturing on CBA. No fluorescent colonies were detected in blood or splenic tissue.

not in the rectum. At 6 h post administration the microorganism was equally present throughout the intestine (stomach, cecum, and rectum) with maximum concentrations of approximately 5×10^{10} CFU/g faeces in all intestinal sections as determined by subculture. At this time-point, viable microorganisms (*e.g. enterococci, E. coli* with and without fluorescence) were subcultured for the first time from at least three homogenized PP and MLN from different locations of the intestine (Fig. 4). No organisms were detected in the spleen, liver or blood of the animals at all time-points. By fluorescent microscopy and immunohistochemistry, it became apparent that the organisms were mainly localized close to the epithelial surface of the stomach, cecum, and rectum and also in the lumen of the intestine (Figs. 5 and 6).

3.2.2. Colonization of rats and intermittent antibiotic challenge

The colonization of Fisher rats by EcN in comparison to EcR was monitored after a 3-day oral antibiotic pre-treatment with ampicillin followed by a 3-day period of oral administration of EcN-GFP or EcR-GFP. Following oral administration of $3–4\times10^{9}$ CFU the numbers of EcN-GFP in faeces steadily declined, until at day 14 faecal cultures for EcN-GFP were negative. In contrast, the numbers of EcR-GFP remained stable. Following renewed administration of ampicillin on days 15–17, concentrations of EcN-GFP and also EcR-GFP increased to reach a maximum of 1×10^{11} CFU/g on day 17. Again, numbers of EcN-GFP declined with time and EcN-GFP was not detectable at day 45, despite administration of

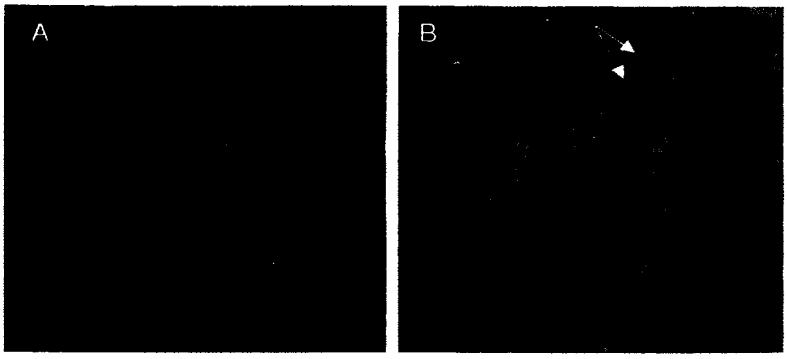

Fig. 5. Fluorescence microscopy of histological sections of the caecum from untreated controls (A, autofluorescence of intestinal tissues) and from mice challenged with EcN-GFP (B). EcN-GFP appears as a bright band close to the epithelial surface on the bottom of the crypt (arrows)(×400).

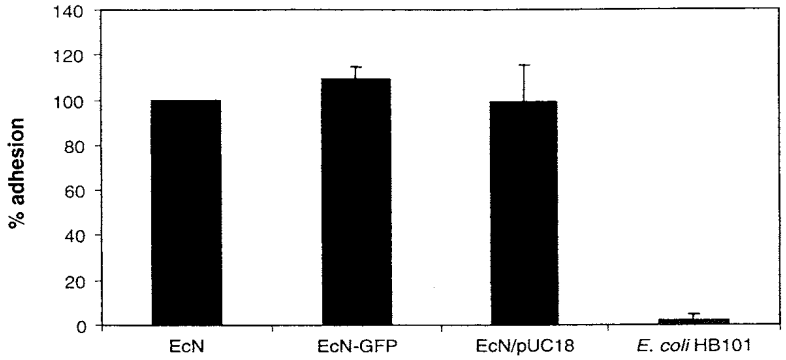

Fig. 2. Adhesion of different bacterial strains to HT-29 epithelial cells. No difference in adherence efficiency was observed with EcN-GFP and EcN containing the empty plasmid vector compared with EcN. *E. coli* HB 101, with no adhesion to epithelial cells, was used as negative control.

3.1.2. In vitro stimulation of IL-8 secretion by HT-29 cells

Secretion of IL-8 from HT-29 cells was stimulated by co-incubation with EcN and EcN-GFP in vitro. IL-8 secretion was dose-dependent with maximum values observed at concentrations of 10^2 to 10^4 CFU/ml. No significant difference in secretion of IL-8 could be demonstrated for stimulation with EcN or EcN-GFP (Fig. 1).

3.1.3. In vitro adherence to HT-29 cells

Adherence to host epithelial tissue was investigated in vitro by co-incubating EcN and EcN-GFP with HT-29 cells for 1–3 h. No alteration of in vitro adhesion of EcN-GFP in comparison to EcN to HT-29 cells was noted (Fig. 2).

3.2. Experiments in vivo

3.2.1. Intestinal passage and localisation of EcN-GFP in vivo

We analysed the kinetics of the intestinal passage of EcN-GFP following oral administration in more detail in Balb/c mice. After a single dose of EcN-GFP, fluorescent colonies were detectable in subcultures of faecal samples on CBA plates (Fig. 3). One hour after ingestion, EcN-GFP was detectable in high numbers in the stomach and in lower numbers in the caecum, but

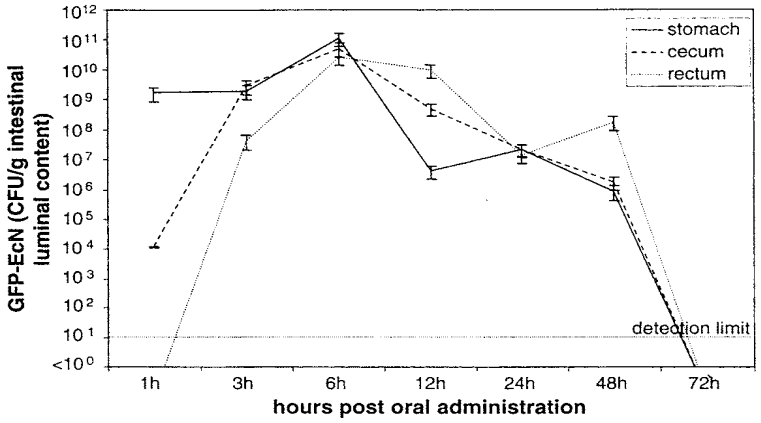

Fig. 3. Intestinal passage of EcN-GFP following single oral administration. EcN-GFP was readily detectable in intestinal luminal content, cultured on CBA up to 48 h.

2.2.4. Typing of E. coli isolates by repetitive extragenic palindromic PCR (REP-PCR)

REP-PCR was performed according to Versalovic et al. (1991). Briefly, after adjustment of the concentration of genomic DNA, PCR was performed using primers REP-1 5′-GCGCCGICATCAGGC-3′ and REP-2 5′-ACGTCT-TATCAGGCCTAC-3′ with 30 cycles of 94 °C for 1 min, 40 °C for 1 min and 65 °C for 8 min. Patterns with different bands were considered to represent different *E. coli* strains.

2.2.5. Detection of EcN-GFP by fluorescence microscopy

Tissue sections from the stomach, jejunum, ileum, caecum, colon, and rectum were embedded (Tissue Tek®) and frozen in liquid nitrogen using an *iso*-pentane heat buffer to prevent snap-freezing and tissue damage. Frozen samples were stored at −70 °C until further use. Frozen sections (3–4 μm thick) and H+E stained sections were examined using native contrast and fluorescent light. Images were taken with a digital camera (RT Color Spot 2.2.1., Diagnostic Instruments Inc., St. Sterling Heights, MI).

2.3. Statistical analysis

For statistical analysis, Sigma Stat 2.03 (SPSS Inc., Chicago, IL 60606 USA) software was used. To control for normally distributed variables, the Kolmogorow–Smirnow test was used, followed by a *t*-test. A $p<0.05$ was regarded as significant, while higher values were regarded as non-significant (ns).

3. Results

3.1. Experiments in vitro

3.1.1. Stability of EcN-GFP in vitro

Transformation of EcR and EcN with plasmid pUC-*gfp* resulted in clones EcR-GFP and EcN-GFP with uniform and bright display of fluorescence of single colonies after 24 h on LB agar plates containing 100 mg/l ampicillin. Daily serial subculture of EcN-GFP on Mueller–Hinton-agar without ampicillin demonstrated that after 14 days more than 50% of approximately 1000 CFUs harboured the plasmid, as evidenced from fluorescence. Subcultures of non-fluorescent colonies failed to grow on Mueller–Hinton-agar supplemented with ampicillin. Under the microscope at ×1000 magnification and fluorescent light, single fluorescent bacterial cells with different intensity of fluorescence could be detected (data not shown).

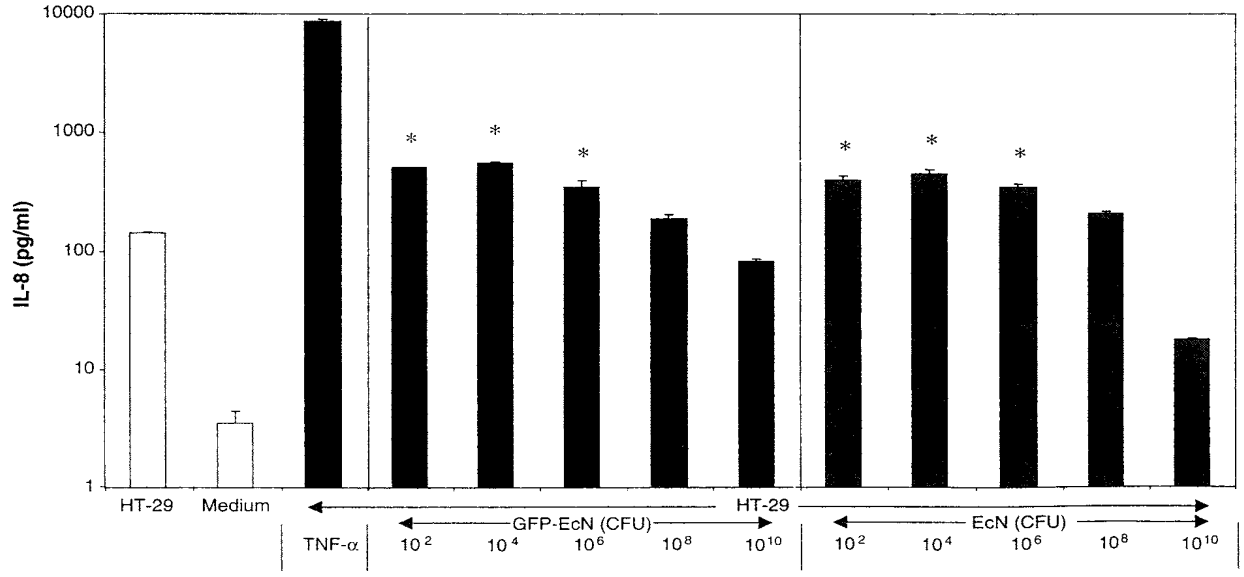

Fig. 1. Comparison of the ability of EcN and EcN-GFP to stimulate the secretion of IL-8 in HT-29 epithelial cells. There was no significant difference between the original EcN and EcN-GFP. *$p\leq0.05$ vs. unstimulated HT-29 cells. Shown is one of at least three experiments.

detected using an UV lamp (Micro-Bio-Tec, Gießen, Germany; 365 nm, 2×4 W) or a fluorescence microscope (Leica DMR X, Leitz, Wetzlar, Germany; fluorescence lamp Osram HBO 100 W, excitation filter 450–490 nm, band-elimination filter 515–560 nm). EcN-GFP was cured from the plasmid (EcN-cGFP) by repeated subculture to antibiotic-free media until disappearance of fluorescence and loss of resistance to ampicillin.

2.1.2. Stimulation of HT-29 cells and determination of interleukin (IL)-8 secretion

HT-29 intestinal epithelial cells at a concentration of 200,000/well of a 24 well plate were incubated at 37 °C in room air supplemented with 10% CO_2 for 24 h in antibiotic-free Dulbecco's MEM medium (GIBCO/BRL Invitrogen, Karlsruhe, Germany, 1 g/l D-glucose; 3.7 g/l $NaHCO_3$; 1.02 g/l *N*-acetyl-L-alanyl-L-glutamine) supplemented with 10% fetal calf serum (FCS; PAN Biotech, Aidenbach, Germany), non-essential amino acids, and 1% Na-Pyruvate (Biochrom, Berlin, Germany). Following renewal of the medium, co-incubation of EcN or EcN-GFP at concentrations from 1×10^2–1×10^{10} CFU/ml and HT-29 cells was performed for 16 h, and IL-8 was determined in the supernatant by ELISA (EH2-IL-8; Endogen, Perbio Science, Bonn, Germany). Viability of the cells was determined by trypan blue exclusion. As positive control, stimulation of the cells with 125 μg TNF-α/well (R and D Systems, Wiesbaden-Nordenstadt, Germany) was performed. All experiments were performed in duplicate and repeated three times.

2.1.3. Adhesion assay

To test for the influence of the labelling of EcN-GFP on adhesion to HT-29 intestinal epithelial cells, a standard adhesion assay was used (Boudeau et al., 2003). Bacteria (EcN, EcN-GFP, *E. coli* HB101 (as control)) were cultivated in liquid LB broth over night at 37 °C without agitation. Of a dilution (A_{600}=0.01) from the overnight culture, 8 μl were used to infect a confluent HT-29 monolayer in a well of a microtiter plate. Confluency of the HT-29 cell monolayer was reached after 24 h incubation under cell culture conditions. The infected HT-29 cells were incubated under cell culture conditions for 2 h. Each well was subsequently washed with 100 μl EBSS (Earl's balanced salt solution) three times. Addition of 100 μl Triton X-100 and shaking for 20 min resulted in lysis of the epithelial cells. Appropriate dilutions of the lysate were plated on LB agar plates. The number of HT-29 associated CFU was counted (Oelschlaeger and Tall, 1997).

All experiments were repeated at least three times and three wells per condition were used.

2.2. Experiments in vivo

2.2.1. Intestinal distribution and kinetics of EcN-GFP in mice following oral administration

Commercially available Balb/C mice at 8–12 weeks and 18–20 g body weight (Charles River, Sulzfeld, Germany) were orally inoculated with a single-dose of 200 μl sterile saline containing 5×10^{10} CFU/ml EcN-GFP. At 1, 3, 6, 12, 24, 48, and 72 h post gastric lavage, two animals and one control animal were sacrificed by CO_2 asphyxation. Liver, spleen and all detectable MLN and PP were removed aseptically before the intestine was opened. Cardiac blood was taken and used undiluted. To minimize the risk of contamination, the intestinum was opened at the end of the procedures to take samples from luminal contents from the stomach, cecum, and rectum. All samples were weighed, homogenized, serially diluted in saline, and cultured on CBA and MacConkey plates (Merck, Darmstadt) overnight at 37 °C in air supplemented with 5% CO_2. Numbers of CFU were normalized by weight.

2.2.2. Immunohistochemistry for EcN-GFP

Intestinal tissue was washed vigorously in sterile PBS, embedded (Tissue Tek® O.C.T. compound 4583; Zoeterwoude, The Netherlands), and frozen in liquid nitrogen using an *iso*-pentane heat buffer to prevent snap-freezing. Cryosections of intestinal tissue were first blocked with FCS and then incubated in PBS containing anti-GFP rabbit polyclonal IgG antibody. As secondary antibody, an anti-rabbit IgG-HRP was used (Santa Cruz Biotechnology, Santa Cruz, CA), according to the manufacturer's instructions of the VECTOR®NovaRED substrate kit (Vector Laboratories, Burlingame, CA).

2.2.3. Colonization of rats and intermittent antibiotic challenge

Commercially available Fisher rats (Charles River) (n=4) were treated with ampicillin (50 mg/kg body weight) in drinking water for 3 days and then challenged on three consecutive days by gastric lavage with 1000 μl PBS containing $3–4\times10^9$ CFU of EcN-GFP or EcR-GFP. Stool samples (1 g wet weight) were collected on days 0, 3, 7, 10, 14, 17, 23, 30, 37, and 45, diluted and homogenized in 100 μl sterile saline. Serial dilutions were cultured on blood agar plates at aerobic conditions. After 24 h, plates were inspected under UV light. As soon as the absence of fluorescent colonies from faecal samples was noted (day 14 and day 37), rats were given ampicillin (50 mg/kg body weight) (days 15–17 and days 38–40) or clindamycin (50 mg/kg body weight; days 31–33) in drinking water, followed by culture of the faecal flora.

reappearing fluorescent colonies. The plasmid was not stable in vivo since non-fluorescent EcN colonies were detected also in faecal samples by REP-PCR.

In summary, transformation of EcN to obtain EcN-GFP in our study had no detectable influence on the probiotic microorganism regarding adhesion on and induction of IL-8 secretion of HT-29 cells and allows the detection in mixed microbial environments in vivo but the stability of EcN-GFP in vivo is limited.

Keywords: Probiotics; Green fluorescent protein; *E. coli* Nissle 1917

1. Introduction

Probiotic microorganisms, such as different Lactobacilli, Bifidobacteria, and Enterobacteriaceae, are defined as viable nutritional agents conferring benefit to the health of the human host (Lilly and Stilwell, 1965). Clinically, beneficial effects of probiotics have been demonstrated for the treatment of infectious diarrhea in infants (Isolauri, 2003), amelioration of side-effects of antibiotic therapy (Cremonini et al., 2002), prevention of allergies (Bienenstock et al., 2002), and treatment of inflammatory bowel diseases (IBD) (Schultz et al., 2003a,b). Especially, *Escherichia coli* strain Nissle 1917 (EcN) was shown to be equally effective as mesalazine in the maintenance of remission in ulcerative colitis (UC) (Kruis et al., 1997, 2004; Rembacken et al., 1999).

The detailed mechanisms by which probiotic bacteria mediate their effects are beginning to be elucidated (Rachmilewitz et al., 2004) but are still not fully understood and seem complex and *diverse* among different probiotic preparations (Bai et al., 2004; Ulisse et al., 2001). Studies in humans, animals, and in vitro suggest that probiotic bacteria modulate the composition of the intestinal microflora (Tannock et al., 2000) or enhance the intestinal mucosal barrier (Mack et al., 1999; Madsen et al., 2001). A direct, anti-inflammatory influence on the intestinal immune system has also been suggested (Rachmilewitz et al., 2004; Malin et al., 1996; Schultz et al., 2003a,b).

Further in vitro experiments have suggested that probiotic bacteria are able to immunomodulate the cytokine response of intestinal mucosal cells in response to luminal antigens (Borruel et al., 2003), but in vivo confirmation with regard to the complex relationships involving possible interspecies and intraspecies interactions with orally administered probiotic bacteria are rare (Boudeau et al., 2003; Mack et al., 1999).

Presumably, the therapeutic effect of a probiotic organism can be linked to its presence in the region of interest. Especially with the probiotic EcN it remains difficult to follow the orally administered strain on its passage through the complex microbial environment of the intestine in vivo, inhabited dominantly by different *E. coli* strains, using traditional culturing methods (Simon and Gorbach, 1984).

For the present report, we transformed EcN with a high-copy number plasmid carrying a modified *gfp* gene to obtain EcN-GFP, suitable for in vivo detection in mixed cultures or in histological sections. We further analysed the impact of labelling on strain-specific properties in vivo and in vitro and addressed questions regarding colonization kinetics and host cell interaction.

2. Methods and materials

2.1. Experiments in vitro

2.1.1. Bacterial strains and transformation

EcN was obtained from Ardeypharm GmbH, Herdecke, Germany. A wild-type strain of *E. coli* (EcR) was cultured from the faeces of a laboratory rat. The isolates were maintained on Columbia blood agar (CBA) and MacConkey agar plates (Merck, Darmstadt, Germany). Transformation of EcN and EcR to obtain EcN-GFP and EcR-GFP with plasmid pUC-*gfp* was performed using standard techniques (Sambrook et al., 1989). In brief, pUC-*gfp* was derived from the high-copy number vector pUC18 by insertion of a modified *gfp* gene under control of the *lac* repressor, allowing single cell detection by fluorescence microscopy (Crameri et al., 1996). Bacterial cells harbouring the plasmid were maintained on Mueller–Hinton-agar (Merck) or in Luria–Bertani broth (LB; Merck) in the presence of 100 mg/l ampicillin (Ratiopharm, Ulm, Germany). Fluorescence was

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Green fluorescent protein for detection of the probiotic microorganism *Escherichia coli* strain Nissle 1917 (EcN) in vivo

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Abstract

Probiotic microorganisms are defined as viable nutritional agents conferring benefit to the health of the human host. Especially, *Escherichia coli* strain Nissle 1917 (EcN) was shown to be equally effective as mesalazine in the maintenance of remission in ulcerative colitis (UC). Presumably, the therapeutic effect of EcN is linked to the presence of the strain in the region of interest; however, it remains difficult to follow the orally administered strain on its passage through the complex microbial environment of the intestine in vivo, inhabited dominantly by various *E. coli* strains, using traditional culturing methods. In this study we transformed EcN and a wild-type *E. coli* from a laboratory rat (EcR) with a plasmid carrying a *gfp* gene (pUC-*gfp*) to obtain EcN- and EcR-GFP to allow in vivo detection without alteration of strain-specific characteristics. Analysis of different strain-specific characteristics included the measurement of stimulation of IL-8 secretion and adhesion in vitro using the epithelial cell line HT-29. The kinetics of intestinal distribution in mice and colonization properties in rats following oral administration was studied in vivo. Detectability of the strain in histologic specimens was analysed using fluorescence microscopy and immunohistochemistry. The identity of fluorescent *E. coli* strains isolated from stool samples, Peyer's patches (PP) and mesenteric lymph nodes (MLN) was determined by REP-PCR. We were able to demonstrate that EcN and EcN-GFP do not differ in stimulation of IL-8 secretion or adhesion to HT-29 cells. In vivo, EcN-GFP colonies were readily detectable by fluorescence microscopy in luminal samples and also by immunohistochemistry in histological sections allowing analysis of the kinetics of the intestinal passage following oral administration. Translocation of fluorescent and non-fluorescent bacteria into PP and MLN was noted at 6 h post oral administration. EcN-GFP was detectable initially for 14 days in faecal samples of rats, while EcR-GFP was detectable throughout the whole experiment (45 days). Challenge with ampicillin at day 45 demonstrated continuing presence of EcN-GFP in small numbers by

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